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cDNA Sequences Reveal mRNAs for Two Gα Signal Transducing Proteins from Larval Cilia

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Abstract. In planktonic larvae of the gastropod mollusk, *Haliotis rufescens* (red abalone), settlement behavior and subsequent metamorphosis are controlled by two convergent chemosensory pathways that report unique peptide and amino acid signals from the environment. The integration of signals from these two sensory pathways provides for variable amplification, or fine-tuning, of larval responsiveness to the inducers of settlement and metamorphosis. These pathways may be analogous to the neuronal and molecular mechanisms of facilitation and long-term potentiation characterized in other (adult) molluscan systems. Recently, the chemosensory receptors and signal transducers apparently belonging to the regulatory pathway (including a G protein and protein kinase C) have been identified in cilia purified from *H. rufescens* larvae. These elements retain their sequential receptor-dependent regulation in the isolated cilia *in vitro*. As a first step toward the molecular genetic dissection of the receptors, transducers, and the mechanisms of their control of settlement behavior and metamorphosis, we present evidence that the cilia purified from these larvae contain polyadenylated mRNA corresponding to unique signal transducers. Purification of this mRNA, enzymatic synthesis of the corresponding cDNAs, amplification by the polymerase chain reaction, cloning, and sequence analysis reveal that the ciliary mRNA includes sequences that apparently code for two Gα signal transducing proteins. One of these is highly homologous to members of the G_q family, recently shown in other systems to control the activity of phospholipase C; the other is more closely related to G_i and G_o. These results extend the tractability of the *Haliotis* system to analyses of cDNA and protein sequences of chemosensory elements from isolated cilia. This is the first time that mRNA has been purified from isolated cilia, and the corresponding cDNA synthesized and characterized.

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[1] Abbreviations: GITC, guanidinium isothiocyanate; SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-thiogalactoside; X-gal, bromo-, chloro-indolylgalactoside; AMV, avian myeloblastosis virus; Tris, tris-hydroxymethylaminomethane; EDTA, ethylenediamine-tetra-acetic acid; TE, Tris-EDTA; TBE, Tris-borate-EDTA; TAE, Tris-acetate-EDTA; bp, base-pair(s). The standard one-letter amino acid code is used.

Introduction

Larvae of the gastropod mollusk, *Haliotis rufescens* (red abalone), undergo a dramatic behavioral change when they encounter a specific chemical cue at the surfaces of crustose red algae: the planktonic larvae cease swimming, attach to the algal surface, and commence metamorphosis and plantigrade locomotion and feeding. This behavioral transition is controlled by the integration of two convergent chemosensory pathways that respond to chemical signals from the environment: a *morphogenetic pathway* activated by a GABA-mimetic morphogen encountered by the larvae on surfaces of recruiting algae, and a *regulatory* or *amplifier pathway* stimulated by lysine in seawater (Morse *et al.*, 1984; Trapido-Rosenthal and Morse, 1985, 1986a, b; Baxter and Morse, 1987; Morse 1990a). Activation of the morphogenetic pathway receptors is thought to trigger an efflux of chloride or other anions across the membrane of the primary chemosensory cell, apparently resulting in excitatory depolarization (Baloun and Morse, 1984). This transduction of the exogenous chemical signal to one that can be propagated by the larval nervous system is evidently sufficient to induce the change in larval behavior culminating in settlement, attachment, and the start of metamorphosis. Activation of the amplifier pathway receptors increases the sensitivity or output of the morphogenetic pathway by as much as 100-fold. The

receptors of the amplifier pathway are activated when they bind lysine, lysine polymers, or certain lysine analogs (Trapido-Rosenthal and Morse, 1985, 1986b). Experiments *in vivo* demonstrated that the amplifier pathway is controlled by chemosensory receptors and signal transducers distinct from those of the morphogenetic pathway, and that the lysine receptors of the amplifier pathway activate a sequential G protein-(phospholipase C) diacylglycerol-protein kinase C signal transduction cascade (Baxter and Morse, 1987). This system of dual control, in which the integration of two different kinds of chemosensory signals from the environment modulates the settlement behavior of the *Haliotis* larvae, fine-tunes larval responsiveness to exogenous settlement cues. The result of this integration may enhance the site-specificity of larval settlement and metamorphosis in potentially favorable habitats (Trapido-Rosenthal and Morse, 1985, 1986b; Morse, 1990a, b).

Because both the morphogenetic and amplifier pathways can be activated by macromolecular (protein-associated or polypeptide) ligands that are presumably impermeant (Morse *et al.*, 1984; Trapido-Rosenthal and Morse, 1985, 1986b), it was suspected that the chemosensory receptors controlling these two pathways might be located on externally accessible epithelia (Morse, 1985, 1990a, b). Epithelial cilia are known to carry chemosensory receptors in a wide variety of systems, including the well-characterized olfactory epithelia of frogs, fish, and mammals (*e.g.*, Rhein and Cagan, 1980; Chen and Lancet 1984; Pace *et al.*, 1985; Pace and Lancet, 1986; Lancet and Pace, 1987; Anholt, 1987; Anholt *et al.*, 1987). Epithelial cilia also have long been suspected to carry the chemosensory structures that mediate substratum recognition and thereby control settlement behavior and metamorphosis in various molluscan larvae (Raven, 1958; Fretter and Graham, 1962; Bonar, 1978a, b; Chia and Ross, 1984; Yool, 1985).

Recently, epithelial cilia isolated from *H. rufescens* larvae were shown to contain the lysine receptors and signal transducers that may control the amplifier pathway *in vivo* (Baxter and Morse, in prep.). These elements retain their functional coupling in the isolated cilia *in vitro*: *i.e.*, the specific and saturable binding of lysine to sodium-independent lysine receptors activates sequentially a G protein and diacylglycerol-stimulated protein kinase C (Baxter, 1991; Baxter and Morse, in prep.). The lysine-binding receptor was found to be reciprocally regulated by its tightly coupled G protein in the cilia *in vitro* (Baxter and Morse, in prep.); similar behavior is exhibited by other members of the rhodopsin and β-adrenergic G protein-coupled transmembrane receptor superfamily.

The tools of molecular genetics are required to further resolve the mechanisms by which the chemosensory and neuronal receptors, transducers, and pathways are integrated to control behavior in these small larvae (Morse, 1990a). As a first step toward that objective, we report here the amplification, cloning, and partial sequence analysis of cDNAs apparently corresponding to two Gα signal transducing proteins, from mRNA purified from the isolated cilia.

Materials and Methods

Cilia isolation

Larvae of *Haliotis rufescens* were produced in the laboratory by hydrogen peroxide-induced spawning of gravid adults (Morse *et al.*, 1977). Larvae were maintained at 15°C in 5 μm-filtered, UV-sterilized running seawater until 7 days post-fertilization; at this time they become developmentally competent to metamorphose in response to inducer (Morse *et al.*, 1979, 1980). Cilia were purified by differential centrifugation, after abscision induced by exposure of the larvae to a mild calcium-ethanol shock (Baxter and Morse, in prep.). This method is a modification of that used for the purification of functional receptor-bearing cilia from olfactory epithelia (Rhein and Cagan, 1980; Chen and Lancet, 1984) and other sources (Watson and Hopkins, 1962; Linck, 1973). Electron micrograph examination reveals the purified cilia to be intact and completely free of cell bodies and debris; the cilia are heterogeneous, and include short (*ca.* 0.5 μm) spatulate cilia similar to the sensory cilia found in other invertebrate systems, and long (≥10 μm) propulsive cilia from the larval velum (Baxter and Morse, in prep.).

RNA isolation

We isolated total RNA from cilia freshly purified from *Haliotis rufescens* larvae, using a single-step extraction with an acid guanidinium isothiocyanate (GITC[1])-phenol-chloroform mixture (Chomczynski and Sacchi, 1987). Poly A^+ mRNA was purified using oligo (dT) cellulose columns either centrifuged (Clontech, Palo Alto, California) or used with a syringe (Stratagene, La Jolla, California) in a modification of the technique described by Aviv and Leder (1972).

RNA was purified from bovine retina to provide a positive control enriched for G protein (transducin) mRNA. For this purpose, fresh bovine eyes were obtained from Federal Meat Market (Vernon, California). Retinas were immediately dissected on ice in Tris-buffered saline (Maniatis *et al.*, 1982) and placed on dry ice for transport to a nearby laboratory. There, half the samples were frozen in liquid nitrogen, and half were homogenized in GITC; these samples then were transported to Santa Barbara in GITC or on dry ice. RNA was isolated by the GITC-cesium chloride centrifugation method (Chirgwin *et al.*, 1979), and mRNA was then purified as described above.

Total RNA from both sources was analyzed by electrophoresis on formaldehyde gels (Maniatis *et al.*, 1982) with ethidium bromide added to the sample buffer, or was denatured at 65°C for 15 min, quickly chilled on ice, electrophoresed on 1% agarose/TBE gels (Han *et al.*, 1987) and visualized by UV-excited fluorescence after ethidium bromide staining (Maniatis *et al.*, 1982). Purity of total and poly A^+ RNA was confirmed by the ratio of absorbances at 260 and 280 nm and concentrations estimated from the A_{260}.

Synthesis of cDNA and oligonucleotide primers

Reverse transcriptase from avian myeloblastosis virus (AMV) (Invitrogen, San Diego, CA) was used to synthesize first strand cDNA. Cilia mRNA (100–500 ng) or bovine mRNA (1 μg) was used for each 50 μl reaction.

For polymerase chain reaction (PCR) amplifications, two kinds of oligonucleotide primers were made: degenerate primers (D) were used for the first amplifications of the cDNA, and (once the exact sequence of the Gα cDNA was determined) specific primers (S) were used to amplify the genomic sequences from sperm DNA. Oligonucleotides used as primers for PCR were synthesized (as the trityl-derivatives) by an automated oligonucleotide synthesizer (Applied Biosystems Inc., Foster City, California). To reduce the degeneracy of the primers, *Haliotis rufescens* condon usage frequencies (Groppe and Morse, 1989) were taken into consideration, and two separate pools of the downstream primer were synthesized (D_2 and D_3). Degenerate oligonucleotide primer sequences corresponding to the conserved *G* and *G′* domains (Lochrie and Simon, 1988) of G protein α subunits are: D_1: 5′ GAAGGATCCAAGTGGATCCA(GC)TG(CT)TTT 3′; D_2: 5′ CTCAAGCTTTCCT(TG)CTT(AG)TT(TG)AG-(AG)AA 3′; D_3: 5′ CTCAAGCTTTCTT(TG)CTT(AG)-TT(CA)AG(AG)AA 3′. D_1 corresponds to the conserved *G′* domain amino acid sequence, KWI(HQ)CF; D_2 and D_3 correspond to the conserved *G* domain sequence FLNK(KQ)D. Amino acid sequences chosen for these domains were based on the findings of Strathmann *et al.* (1989), with inclusion of a degeneracy representing additional sequences determined for yeast. In addition, these primers include oligonucleotide sequences (indicated by underlining) corresponding to the BamH I (D_1) and Hind III (D_2 and D_3) restriction enzyme targets; these sequences were added as linkers to facilitate cloning of the amplified products. A shorter variant of D_1 also was produced without the 9-nucleotide linker. Specific (non-degenerate) primer sequences based on the cDNA sequence subsequently determined for the *Haliotis* cilia Gα1 (see below) are: S_1: 5′ GCAGGATCCACGTCCATCATGTTCTTA 3′; and S_2: 5′ CTCAAGCTTCGGGTAGGTGA-TAATCGT 3′. These primers also have 9-nucleotide long 5′-linkers with a BamH I site (S_1) or a Hind III site (S_2).

After synthesis, oligonucleotides were deprotected at 55°C in ammonia overnight; they were then purified and detritylated by reverse-phase chromatography (Oligonucleotide Purification Cartridges from Applied Biosystems). The oligonucleotides were dried down, resuspended in sterile water, and concentration was estimated by absorbance at 260 nm.

PCR amplification

For amplification of cDNA, 50 pmol of each primer and 40% (20 μl) of the reverse transcription reaction were added directly to 100 μl PCR reactions. All other reaction components, including Taq DNA polymerase, were purchased from Perkin Elmer-Cetus Corp. (Norwalk, Connecticut) and used as suggested by that manufacturer. The number of amplification cycles was varied from 25 to 45 with the following parameters: denaturation at 94°C, 1 min; annealing at 37°C, 1 min; extension at 72°C, 3 min. For amplification of genomic DNA using specific primers, 0.3 μg *Haliotis rufescens* sperm DNA, 0.75 μg *Tetrahymena thermophila* DNA, 0.5 μg *Vibrio harveyi* DNA, or 1 μg salmon sperm DNA were added to otherwise identical amplification reactions. (The *Haliotis*, *Tetrahymena*, and *Vibrio* genomic DNA samples were generously provided by Jay Groppe, Jennifer Ortiz, and Richard Showalter, respectively.) All DNA samples were tested for amplification at amounts equal to or greater than the number of genome equivalents of the *Haliotis* DNA. Because DNA samples were in TE buffer, additions were adjusted such that the total amount of TE (and thus the concentration of EDTA) in all PCR reactions was equal. Optimum magnesium concentration, primer concentration, and cycling parameters were determined empirically. For amplification of genomic DNA, 25 pmol of each specific primer was used; the final concentration of magnesium was increased from the standard 1.5 m*M* to 2 m*M*; and a total of 30 amplification cycles was performed as before, except that the annealing temperature was raised to 45°C for the 2nd cycle and to 55°C for the 3rd–30th cycles. A 5 min extension step (72°C) was added after the last cycle.

Gel electrophoresis for analysis and purification of PCR reaction products

PCR reaction products were analyzed by electrophoresis on agarose gels (3% Nuseive agarose plus 1% Seaplaque agarose in TBE for cDNA-PCR reactions and 1.5% agarose/TAE buffer for genomic reactions), run at 2–6 v/cm, and stained with ethidium bromide. For analysis on the higher percentage gels, samples were loaded into wells cast from 0.7% agarose. One μg of restriction enzyme-digested plasmid (PBR322-BstN 1, from New England Biolabs Inc., Beverly, Massachusetts) was included for molecular weight markers.

For purification, amplified cDNA was electrophoresed on 3% low melting temperature agarose (Mermaid, from Bio 101 Corp., La Jolla), and DNA bands were excised while visualized on a 365 nm light box. DNA then was removed from the agarose by binding to glass beads (Glass Fog, from Bio 101 Corp.). Genomic products were run on 1.5% agarose gels as described above; bands were excised, and DNA was purified by binding to glass beads (Geneclean, from Bio 101 Corp.).

DNA cloning

Purified PCR products and a recombinant plasmid vector (*p*Bluescript KSII+, from Stratagene Corp.) were digested at 37°C for 1 h in 20 μl volumes with HindIII followed by BamHI (enzymes from New England Biolabs). Digested products were purified by gel electrophoresis as described above. The purified, linearized vector (100 ng) then was ligated to an approximately equimolar amount of PCR product insert; this reaction was catalyzed by T4 DNA ligase overnight at 4°C. Controls with no added insert were treated identically. Transformation of recipient bacteria (Epicurian Coli XL-1 Blue, from Stratagene Corp.) was performed by the method of Hanahan (1983), with modifications recommended by Stratagene Corp. After transformation, colonies with recombinant plasmids were identified on the antibiotic-containing agar medium with the chromogenetic substrate, X-gal. Each clone was then subcultured in 5 ml of LB containing ampicillin and tetracycline (37°C, overnight). Plasmid DNA was purified from these cultures after lysis with alkalai, using the miniprep procedure (Maniatis *et al.*, 1982).

Cloned plasmid DNA was digested as above with BamH I and Hind III simultaneously, and separately with BssH II (for which the plasmid has two sites, flanking the BamH I and Hind III sites). Restriction digests were analyzed by agarose gel electrophoresis. Plasmid DNA was purified by centrifugation chromatography (Sephacryl S-400 Miniprep Spun Columns, from Pharmacia, Piscataway, New Jersey).

DNA sequence analysis

Di-deoxy sequencing reactions were performed with modified T7 DNA polymerase (Sequenase II; United States Biochemical, Cleveland, Ohio) and 5′ [α-^{35}S]dATP, 1100 Ci/mmol (Amersham) by procedures modified from Sanger *et al.* (1977). Primers used for sequencing were plasmid primers T7, T3, KS, and SK (purchased from Stratagene) and the specific primers S_1 and S_2, described above. Reactions were analyzed by electrophoresis on 8% polyacrylamide-50% urea wedge sequencing gels. Gels were washed in 10% acetic acid-10% methanol, dried with vacuum at 80°C, and subjected to autoradiography. The autoradiograms were read with a sonic digitizer, and the resulting sequences analyzed with the aid of the Pustell Sequence Analysis software (IBI Macintosh).

Results

PCR amplification of cilia Gα cDNA

Poly A^+ mRNA was isolated from the purified cilia of 7–9-day-old, competent larvae of *Haliotis rufescens*, as described in the Methods. Starting with about 10^6 larvae, typical yields were 300–400 mg (wet weight) cilia, 50 μg total RNA, and 1 μg of poly A^+ mRNA (=2% of total RNA). AMV reverse transcriptase was used to catalyze random-hexamer primed synthesis of first strand cDNA from the purified mRNA, and the resulting mRNA-cDNA duplex was then used as template for PCR amplification with the degenerate primers corresponding to the conserved *G* and *G′* domains, as described in the Methods.

The primers used clearly directed the amplification of a 196 bp product from the cDNA templates prepared from the larval cilia (Fig. 1a). The size of this product is within the range predicted for a Gα cDNA domain lying between the highly conserved *G* and *G′* domains. This result suggests that the cilia purified from *Haliotis rufescens* larvae may contain mRNA coding for a Gα protein.

The positive result shown in Figure 1a allowed us to further optimize the primers used for PCR-amplification. The upstream primer used in the first PCR amplifications was relatively short, consisting of a degenerate pool of 17-mers. This primer only weakly amplified the positive control template, cDNA from bovine retina, a tissue highly enriched for the Gα known as transducin (results not shown). The addition of nine nucleotides containing the sequence of the BamHI restriction endonuclease site to the 5′-end of the 17-mers, to generate the D_1 primers (see Materials and Methods), makes efficient amplification of the control Gα sequence from bovine retina cDNA possible (Fig. 1b). [Similarly, we had found earlier that addition of the nine nucleotides containing the sequence of the Hind III restriction site to the 5′-end of short downstream primers, to generate the 26-mer D_2 and D_3 pools, also significantly enhanced the efficiency of these oligonucleotides as primers. These non-matching nucleotides do not reduce primer specificity. Similar observations have been reported by others (*e.g.*, Mack and Sninsky, 1988).] The results in Figure 1b show the downstream primers in the degenerate pool D_2 are more effective than those in D_3 for detecting and amplifying Gα cDNA sequences from both bovine retina and *Haliotis rufescens* larval cilia. Primer D_3 differs from D_2 by two nucleotides and apparently fails to hybridize efficiently to the target G protein cDNA sequence. Agarose gel electrophoresis of PCR products amplified with the optimized 26-mer primers (D_1 and D_2) reveals the expected 205 bp product from

cDNAs from both the larval cilia and bovine retina. The product is nine nucleotides longer than that seen in Figure 1a, as expected because the upstream primer (D_1) is nine nucleotides longer than that used in the first experiment. The cilia cDNA required more cycles of amplification (35) than did the bovine retina cDNA (25) before the product on an ethidium bromide stained gel could be visualized. Thus, there may be greater primer-template mismatch, or the target mRNA may be less abundant, in the larval cilia.

A control PCR reaction with no added DNA template, a test for DNA contamination of reagents, yielded no detectable PCR products amplified after 45 cycles (Fig. 1b). In addition, no amplified PCR products were observed in the following control reactions (not shown): (a) no primers in the PCR reaction; (b) no Taq polymerase in the PCR reaction; and (c) no reverse transcriptase in the cDNA reaction.

Cloning and sequence analysis of Gα cDNA

The 205 bp PCR product from the cilia cDNA was cloned in the plasmid vector as described in Materials and Methods. Twelve transformant colonies were picked and subcultured; gel electrophoresis of the restriction enzyme-digested plasmid DNA showed that 11 of these clones contained inserts of the correct size.

Sequence analysis of three of the cloned cilia PCR products revealed two unique cDNA sequences (Fig. 2). As shown, both of these share a number of the highly conserved residues of other Gα proteins in the *G–G′* region. Two out of the three clones proved to have identical nucleotide (and deduced protein) sequences over the 51 amino acid region between the primers. This *Haliotis* G protein α subunit (Gα1) differs in the region analyzed by only one amino acid from the sequence of mouse brain Gα11, a member of the newly discovered G_q class of α subunits (Strathmann *et al.*, 1989; Strathmann and Simon, 1990). This sequence differs significantly in the region analyzed from all other known classes of G protein α subunits (G_s, G_t, G_{olf}, G_o, G_i, G_x) from mammals, *Drosophila*, and yeast. The second G protein sequence from the larval cilia (*Haliotis* Gα2) is most homologous to G_o and G_i from *Drosophila*.

Figure 1(a). G protein α subunit cDNA from cilia, amplified by PCR. Product from 50 cycles of amplification; primers were a 17-mer variant of D_1 (without the BamHI site) and D_2. Number of base-pairs in product is indicated. Details in Materials and Methods.

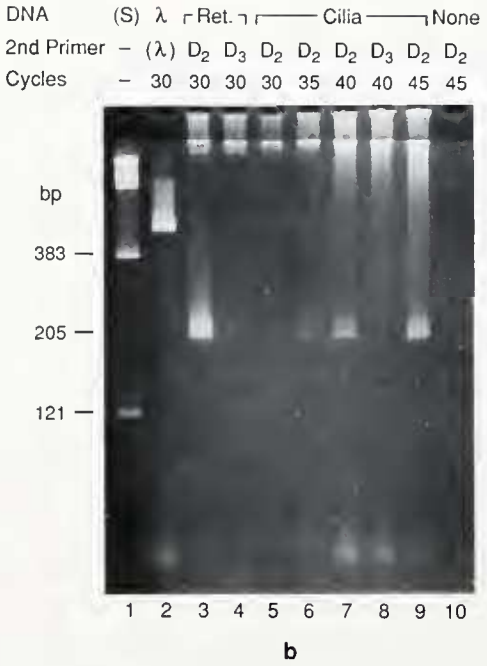

Figure 1(b). PCR amplification of larval cilia cDNA using degenerate primers D_1 and either D_2 or D_3. Lanes: (1) molecular weight standards; (2) λ DNA and primers as PCR control; (3) bovine retina cDNA, primers D_1 and D_2, 30 cycles; (4) bovine retina cDNA, primers D_1 and D_3, 30 cycles; (5) cilia cDNA, primers D_1 and D_2, 30 cycles; (6) cilia cDNA, primers D_1 and D_2, 35 cycles; (7) cilia cDNA, primers D_1 and D_2, 40 cycles; (8) cilia cDNA, primers D_1 and D_3, 40 cycles (9) cilia cDNA, primers D_1 and D_2, 45 cycles; (10) no DNA template, primers D_1 and D_2, 45 cycles.

Amplification, cloning, and sequence analysis of Gα from Haliotis *genomic DNA*

The nucleotide sequence from the *Haliotis rufescens* larval cilia Gα1 clone was used to design specific (*i.e.*, non-degenerate) primers to amplify the corresponding region of Gα from *H. rufescens* sperm genomic DNA. The specific primers (S_1 and S_2) were based on regions of the *Haliotis* sequence that differed significantly from other Gα protein sequences (Fig. 5; *cf.* Fig. 2).

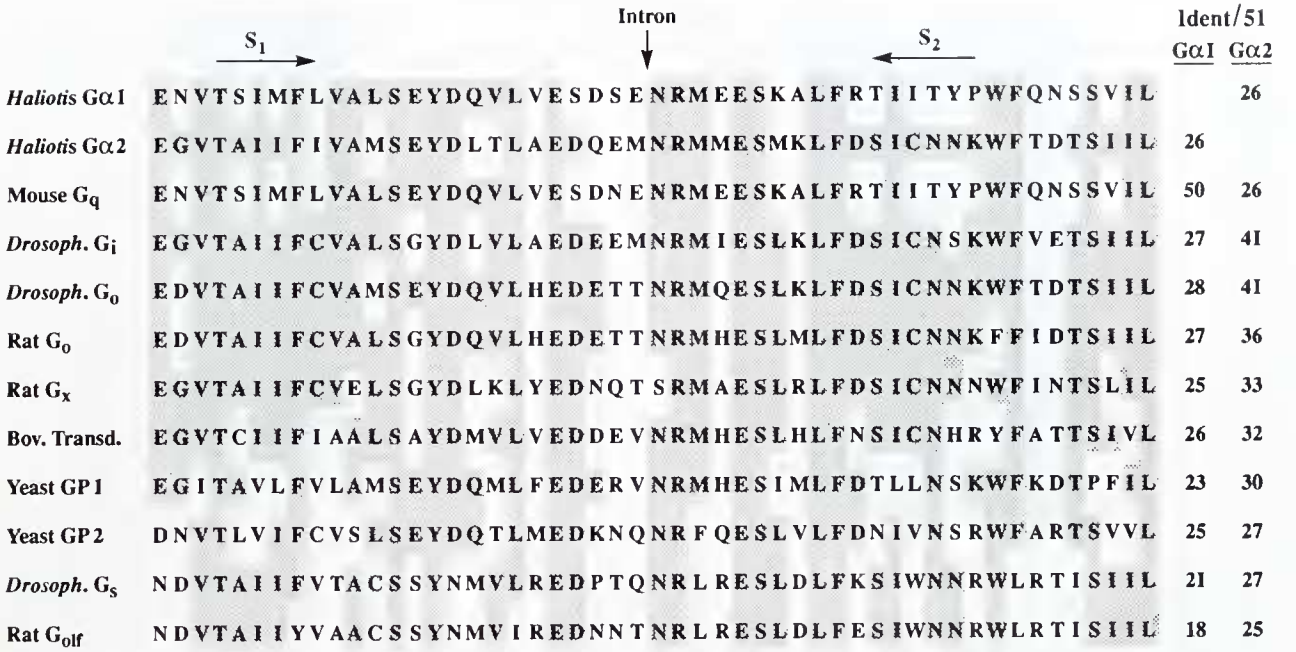

Figure 2. Gα sequences from cilia. Deduced amino acid sequences of the cloned PCR products (Gα1 and Gα2) from *Haliotis rufescens* cilia cDNA are compared with the corresponding regions of other Gα subunits. Residues highly homologous in several Gα proteins are indicated by shading. Region shown is between (not including) degenerate primers D_1 and D_2. The number of amino acids identical to *Haliotis* Gα1 and Gα2 (of 51 total) is shown for each Gα. Sequences used to design specific primers S_1 and S_2 are shown by arrows; position of the intron in genomic DNA is indicated. Standard (IUPAC) one-letter amino acid code is used.

Two distinct DNA products were seen when *Haliotis* genomic DNA was amplified by PCR reactions with the S_1 and S_2 primers, and the products were analyzed on an agarose gel (Fig. 3). The specificity of these primers for *Haliotis* DNA is evident by their failure to direct amplification of genomic DNA sequences from *Tetrahymena, Vibrio,* or salmon sperm in otherwise identical PCR reactions.

The 1.25 kb and 1.45 kb *Haliotis* genomic PCR products were electrophoretically purified, separately amplified again, and analyzed electrophoretically (Fig. 4). The successful purification of the two genomic PCR products is shown by the lack of visible contamination after this second round of amplification and gel electrophoresis. Each purified PCR product was cloned as described above, and the cloned inserts were then sequenced using primers matched to the vector.

During the cloning step we discovered that the larger of the two genomic PCR products had an internal Hind III site not found in the smaller product. This difference apparently resides in an intron, a non-coding sequence not present in the cDNA (see below). Although the resulting Hind III-Hind III fragment was not cloned, a partial sequence for this region was obtained from the direct sequencing of the uncloned PCR products using S_1 and S_2 as primers for the sequencing reaction. While this method of direct sequencing proved useful in this case, the quality of the sequencing reactions was highly variable (*cf.* McCabe, 1989).

The genomic PCR product sequences were identical to the Gα1 cDNA sequence from the cilia, and to one another, in the putative coding regions. But both of the cloned genomic sequences contain an intron that interrupts the coding sequence. The exact position of this intron between exons 6 and 7 is conserved in the genomic sequences corresponding to the *Haliotis* Gα1 and the G_o and G_i of mammals and *Drosophila* (Figs. 2, 5). The two *Haliotis* genomic sequences prove to be highly homologous to one another at both ends of the introns that are adjacent to the coding regions (corresponding to exons 6 and 7 in other species; *cf.* Fig. 5); however, the two introns differ in length by about 200 bp.

Discussion

The larvae of *Haliotis rufescens* provide a uniquely tractable model system for resolving and analyzing the chemosensory receptors and signal transducers, and the mechanisms of their functional integration, controlling behavior and development in response to chemical signals

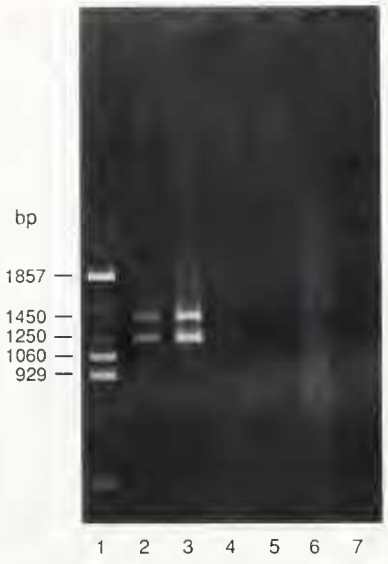

Figure 3. PCR amplification of genomic DNA using *Haliotis*-specific primers S_1 and S_2 (30 cycles). Lanes: (1) molecular weight standards; (2) and (3) 0.3 μg *Haliotis* sperm DNA; (4) 0.75 μg *Tetrahymena* DNA; (5) 0.5 μg *Vibrio* DNA; (6) 1 μg salmon sperm DNA; (7) no DNA. Other details in Materials and Methods.

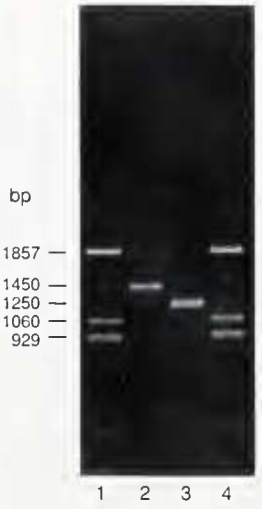

Figure 4. *Haliotis* genomic PCR products after gel purification and 30 additional cycles of amplification with specific primers S_1 and S_2. Lanes: (1) molecular weight standards; (2) *ca.* 1.45 kb genomic PCR product; (3) *ca.* 1.25 kb genomic PCR product; (4) molecular weight standards.

from the environment (Morse, 1990a, b). Purification of cilia in milligram quantities from the cultured larvae has allowed us to analyze the chemosensory receptors and signal transducers *in vitro* (Baxter, 1991; Baxter and Morse, in prep.), and (as shown here) to isolate mRNAs encoding some of these elements and conduct analyses at the cDNA sequence level. We have shown here that cilia purified from *H. rufescens* larvae contain polyadenylated mRNA, and that this mRNA includes sequences corresponding to two Gα signal transducing proteins.

The central role of the G proteins as chemosensory signal transducers was confirmed by *in vitro* studies of olfactory cilia isolated from frog (Pace *et al.*, 1985). Jones and Reed (1989) recently have identified a unique G_{olf} from the ciliated sensory epithelium of the rat olfactory mucosa; the sequence of this G protein α subunit was determined by analysis of its cDNA. G protein also acts as the primary chemosensory signal transducer in taste receptor cells in the frog; this G protein controls the activation of adenyl cylase, with the resulting sequential activation of a protein kinase and membrane depolarization (Avenet *et al.*, 1988). G protein transduction of chemosensory stimulation in catfish controls an inositol triphosphate (protein kinase) cascade (Huque *et al.*, 1987).

Although distal localization of mRNA has been observed in other systems (Merlie and Sanes, 1985; Garner *et al.*, 1988; Kosik *et al.*, 1989), the results reported here are the first of which we are aware in which mRNA has been purified, and the corresponding cDNA synthesized, amplified, cloned, and sequenced from purified cilia. Because the mRNA from which the G protein sequences were identified was extracted from cilia that had been purified and washed by four sequential cycles of differential centrifugation, it was probably not contaminated by free RNA released from the larvae. Electron microscopy shows no other cell fragments or cells contaminating the purified cilia (Baxter, 1991; Baxter and Morse, in prep.). *In situ* hybridization will be required, however, to verify the intraciliary localization of the Gα mRNA. Three independent lines of evidence confirm that the source of

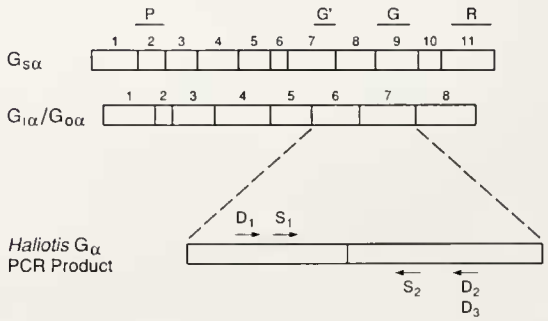

Figure 5. Genomic organization of mammalian G protein α subunits (after Kaziro *et al.*, 1990). Exons (coding regions) are numbered; vertical lines indicate introns. Conserved domains P (GTP hydrolysis), G' and G (GTP binding) and R are shown above. [In the convention of Halliday (1984), domain G also is designated G, but the other domains are designated differently.]. The region of the *Haliotis* G protein corresponding to mammalian G_o and G_i exons 6 and 7 is expanded to show relative positions and orientations of PCR primers. D_1, D_2, and D_3 are degenerate primers used for cDNA amplification; S_1 and S_2 are the *Haliotis*-specific primers used for genomic amplification.

the G protein sequences characterized could not have come from bacterial contamination: (1) the mRNA selected was poly A^+; (2) the sequence of Gα1 determined corresponds exactly to a sequence in the genomic DNA (from sperm) of *Haliotis rufescens;* and (3) that genomic sequence contains an intron (at the expected position) that would be lacking from bacterial DNA. That these sequences were from *Haliotis* mRNA, and not from possible contaminants, was further confirmed by the failure of the unique ciliary sequences to direct amplification of any homologous sequences in control samples of bacterial, protozoan, or fish DNA, under conditions in which the perfectly homologous sequences were detected and amplified from *Haliotis* genomic (sperm) DNA.

The cDNA sequences that we obtained from mRNA purified from the cilia isolated from *Haliotis rufescens* larvae reveal two apparent G protein α subunit sequences. Although definitive characterization must await completion of the entire translated sequences, our identification of these sequences as members of the G protein family is strengthened by the finding that Gα1 is virtually identical (50/51 residues) to $G_q\alpha$ in the region sequenced, and the observation that the most highly conserved amino acids in this region of the other known Gα proteins also are conserved in the Gα1 and Gα2 sequences from the *Haliotis* larvae (Fig. 2). The genomic sequences that we thus far have characterized correspond exactly to their cDNAs, and contain the intron at the same position between the *G* and *G'* domains as those found in mammalian G protein genes.

The two Gα sequences obtained from the cilia of *Haliotis* larvae are clearly related to the Gα sequences from other species (Fig. 2) (and unrelated to tubulin, for example). Yet the two sequences differ significantly from one another. The larval cilia Gα1 sequence is highly homologous to that of the corresponding domain of the alpha subunit of the mammalian G_q. This is of particular interest, in light of the finding that G_q is a pertussis toxin-insensitive regulator of phospholipase C (Strathmann and Simon, 1990; Smrcka *et al.,* 1991), and our observation that the lysine-dependent regulatory pathway in *Haliotis* larvae is mediated by a pertussis toxin-insensitive G protein-phospholipase C-protein kinase cascade (Baxter and Morse, 1987; Baxter, 1991; Baxter and Morse, in prep.). Gα1 is markedly different from G_i, G_s, G_o, G_x, and G_{olf} characterized from other systems, whereas the larval cilia Gα2 sequence is significantly more closely related to G_i and G_o (from *Drosophila* and rat) than it is to the G_s and G_{olf} from these species. We are now in the process of identifying the Gα sequence corresponding to the transducing protein specifically activated by the lysine receptors in the isolated cilia. These experiments are facilitated by the observation that lysine binding to the ciliary receptors activates the associated Gα protein, increasing its radioactive labeling with ADP-ribose catalyzed by cholera toxin (Baxter and Morse, in prep.).

In molluscan larvae, the cilia of the cephalic apical tuft and of the propodium have been suggested to mediate chemosensory substratum-recognition and the resulting control of metamorphosis (Raven, 1958; Fretter and Graham, 1962; Bonar, 1978a, b; Chia and Koss, 1984; Yool, 1985). Ciliary receptors have been implicated in this process in other invertebrate larvae as well (*e.g.,* Laverack, 1968; Carthy and Newell, 1968; Chia and Spaulding, 1972; Siebert, 1974; Zimmer and Wollacott, 1977; Eckelbarger, 1978; Reed and Cloney, 1982; Reed, 1987). The ciliated cells of the apical tufts of *Haliotis* larvae are stellate neurons that appear anatomically to be primary chemosensory receptor cells (Yool, 1985). These neurons project axons to the cephalic ganglia; they also send lateral dendrites to a pair of adjacent mucus gland cells that are stimulated to secrete their contents in one of the first cellular changes observed in metamorphosis (Morse *et al.,* 1980a; Yool, 1985). These neurons and their cilia disappear from the organism after induction of metamorphosis; the time of this loss coincides with the time of loss of the labeled morphogenic receptors (Trapido-Rosenthal and Morse, 1986a), supporting the suggestion that some of the biochemically labeled chemosensory receptors controlling metamorphosis may be located on these cells or their cilia.

Our finding that sufficient mRNA can be obtained from the cilia of *Haliotis* larvae to establish a cDNA library opens this system to analyses, similar to that reported here, of the cDNAs for the receptors and other signal transducers of the morphogenetic and amplifier pathways that control metamorphosis. These analyses should provide insights into the mechanisms of action, functional integration, and evolution of the chemoreceptors and their associated signal-transducers in the molluscan larvae.

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